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TITLE: Chemoprevention of Prostate Cancer Initiation in a Novel Transgenic Mouse Model  
by Targeting 15-Lipoxygenase-1

PRINCIPAL INVESTIGATOR:  
Uddhav P. Kelavkar, Ph.D.

CONTRACTING ORGANIZATION:  
University of Pittsburgh  
Pittsburgh, PA, 15213

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**INTRODUCTION:**

***Fifteen lipxygenase-1 (15-LO-1) in the Mouse Prostate (FLiMP)- A novel mouse model to study impact of omega diets on prostate cancer progression:*** To gain better mechanistic insight of the role of 15-LO-1 in prostate cancer (PCa), we recently developed a novel C57BL/6 transgenic mouse called FLiMP [1]. These mice, which conditionally express prostatic human 15-LO-1, display mouse prostatic intraepithelial neoplasia (mPIN) by week 20, but do not progress to cancer when on normal diet. Therefore, FLiMP mice provide an excellent model to study the experimental paradigm of PCa initiation, highlighting their usefulness in evaluating early “proactive” intervention strategies in PCa. Our proposed studies are predicated on the hypothesis that dietary prevention is an effective means of eradicating PCa, and that FLiMP mice provide a valuable pre-clinical model for chemoprevention studies. Diets rich in either omega ( $\omega$ )-3 or  $\omega$ -6 polyunsaturated fatty acids (PUFAs) directly impact PCa tumor growth. Furthermore, the FLiMP mice, which overexpress human 15-LO-1, faithfully recapitulate the early stages of human PCa progression. These observations support the potential value of  $\omega$ -3 fatty acid SDA as a chemopreventative agent and the need for further studies. Thus, the FLiMP mouse model has the strength of being a genetically defined, immune-competent tool to address the  $\omega$ -6 and  $\omega$ -3 experimental paradigm.

However, during year 2 we had already bred the transgenic FLiMP several times. In order to avoid potential genetic drift in FLiMP mice and variations in expected outcomes, we have begun re-deriving the FLiMP mice. This will take approximately 6-9 months to obtain sufficient male mice to undertake Task 2. We have conserved our budget for this study accordingly. As an alternative, we determined the bioavailability of omega- $(\omega)$ -3 to the tumor, understand mechanisms, and the feasibility of targeting the  $\omega$ -6 polyunsaturated fatty acids (PUFAs) metabolizing 15-lipoxygenase (LO)-1 and cyclooxygenase (COX)-2 pathways. Nude mice injected subcutaneously (s.c) with LAPC-4 prostate cancer (PCa) cells were randomly divided into three different isocaloric (and same percent [%] of total fat) diet groups: high  $\omega$ -6 linoleic acid (LA), high  $\omega$ -3 stearidonic acid (SDA) PUFAs, and normal (control) diets. Tumor growth and apoptosis were examined as end-points after administration of a short-term (5-week)  $\omega$ -3 and  $\omega$ -6 fatty acid diets. Tumors were examined for growth, lipids, enzyme activities, apoptosis and proliferation. Tumors from the LA-diet fed mice exhibited the most rapid growth as compared with tumors from the control and SDA diet fed mice. Moreover, a diet switch from LA to SDA caused a dramatic decrease in the growth of tumors in 5 weeks whereas tumors grew more aggressively when mice were switched from a SDA to LA diet. Evaluating tumor proliferation (Ki-67) and apoptosis (Caspase-3) in mice fed the LA- and SDA-diets suggested increased % proliferation index from the  $\omega$ -6 diet fed mice as compared with tumors from the  $\omega$ -3 SDA-fed mice. Further, increased apoptosis was observed in tumors from  $\omega$ -3 SDA-fed mice versus tumors from  $\omega$ -6 diet fed mice. Levels of membrane phospholipids of red blood cells (RBCs) reflected dietary changes and correlated with the levels observed in tumors. LA or AA and metabolites (eicosanoid/prostaglandins) were analyzed for 15-LO-1 and COX-2 activities by high performance liquid chromatography (HPLC). We also examined the percent (%) unsaturated or % saturated fatty acids in the total phospholipids, PUFA  $\omega$ -6/  $\omega$ -3 ratios and other major enzymes (elongase, delta ( $\Delta$ )-5-desaturase, and  $\Delta$ -6-desaturase) of  $\omega$ -6 catabolic pathways from the tumors. We observed a 2.7-fold increase in the  $\omega$ -6/ $\omega$ -3 ratio in tumors from LA-fed mice and a 4.2-fold decrease in the ratio in tumors from the SDA-fed mice. There was an increased  $\Delta$ -6-desaturase and  $\Delta$ -9 desaturase enzyme activities and reduced estimated  $\Delta$ -5-desaturase activity in tumors from mice fed the SDA-diet. Opposite effects were observed in tumors from mice fed the LA-diet. Together, these observations provide mechanistic roles of  $\omega$ -3 fatty acids in slowing PCa growth by altering  $\omega$ -6/  $\omega$ -3 ratios via diet, and promoting apoptosis and inhibiting proliferation in tumors by directly competing with  $\omega$ -6 fatty acids for 15-LO-1, and COX-2 activities.

We

**BODY:**

Task 2 in years 2-3. Characterize key molecular events altered in prostate cancer initiation in FLiMP<sup>+/+</sup> mouse model by dietary n-6 and n-3 (Months 8-36):

***Experimental Approach:******Cell Culture***

Los Angeles Prostate Cancer-4 (LAPC-4) PCa cells were kindly provided by Dr. Robert Reiter (University of California-Los Angeles) and maintained in phenol red-free DMEM (Invitrogen, Carlsbad, CA) containing 5% heat-inactivated fetal calf serum (FCS, Sigma) with streptomycin-penicillin antibiotics in a 5% CO<sub>2</sub> incubator at 37°C.

***15-lipoxygenase-1 (15-LO-1) and cyclooxygenase (COX) enzyme activities in tumors***

The activities of 15-LO-1 and COX were estimated as described previously by us [2] and expressed as percent conversion of substrate/s to product/s.

***Enzyme activity calculation of other enzymes of eicosanoids pathway***

The activities of enzymes involved in fatty acid biosynthesis were estimated as the product-to-precursor ratios of the percentages of individual fatty acids [3]. The estimated enzyme activities included those of elongase, calculated as the stearic acid (18:0)-to-palmitic acid (16:0) ratio; delta ( $\Delta$ )-5 desaturase, calculated as the arachidonic acid [20:4( $\omega$ -6)]-to-di-homo-gamma ( $\gamma$ )-linolenic acid [20:3( $\omega$ -6)] ratio;  $\Delta$ -6 desaturase, calculated as the di-homo- $\gamma$ -linolenic acid [20:3( $\omega$ -6)]-to-linoleic acid [18:2( $\omega$ -6)] ratio (assuming that  $\Delta$ -6 desaturase and not elongase is rate limiting); and  $\Delta$ -9 desaturase, calculated as the oleic acid [18:1( $\omega$ -9)]-to-stearic acid [18:0 ( $\omega$ -9)] ratio.

***Diets, experimental strategy, feeding Protocol, LAPC-4 cell injection and tumor analyses***

We utilized three different isocaloric diets (caloric density 4.4 kcal/gm) for our study [2]. These custom-made, semi-purified diets were prepared and irradiated by Purina Test Diet, Inc. (Richmond, IN). High  $\omega$ -6 LA and high  $\omega$ -3 SDA fat diet group (experimental) were identical to those that are described before [2]. Use of pure EPA is cost-prohibitive and therefore SDA, an immediate precursor of EPA, served as a legitimate substitute. Furthermore, because of concerns with mercury and polychlorinated biphenyl contamination of fish and fish-oils, which currently provide the major sources of long-chain  $\omega$ -3 in the human diet, land based sources of functional  $\omega$ -3 fatty acids such as SDA are currently being developed [4]. Normal-fat diet (control) was also isocaloric when adjusted by adding dextrin and corn-oil.

Initially we took a total of 101 athymic male BALB/C nude (nu/nu) (6–8 weeks old) mice obtained from Charles River (Wilmington, MA) and fed them initially a no fat diet for 2 weeks as described before [2]. As illustrated in Figure 2, after 2 weeks, we split these mice into 3 groups namely; two experimental groups [a total of n=76 divided into 38 mice per high  $\omega$ -6 LA and  $\omega$ -3 SDA fat diet groups (experimental)] versus the control group (n=25 per normal dietary group). These mice were housed in single sterile animal cages to allow for the maintenance of isocaloric intake between the diet groups. Cages, bedding, and water were autoclaved before use. Specially designed feeding receptacles were placed in the cages so that food intake could be carefully monitored. Sterile techniques were used whenever handling the cages, mice, and food. The Pittsburgh Animal Research Committee approved the experiments, and animals were cared for in accordance with institutional guidelines.

After 2 weeks on no fat diet, all the n=101 mice were injected with LAPC-4 cells. This time point was counted as week 1. The mice were injected in duplicate subcutaneously (s.c.) in the right and left lateral flank with  $1 \times 10^6$  LAPC-4 tumor cells in 0.1 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA). When tumors became palpable, the tumor dimensions were measured [5]. Tumor growth and apoptosis were examined as end-points after administration of different experimental and control short-term (5-week) diets. At week 23, half of the mice from each PUFA group were switched to the opposing diets (i.e., from SDA to LA diet and vice versa) while the other half of the mice remained on their original diet. Tumors were examined for growth, lipids, enzyme activities, apoptosis and proliferation indices.

### ***Red blood cells membrane (RBC's) and tumor phospholipid content analyses***

Phospholipid content from RBC's and tumor tissues membranes were analyzed by temperature-programmed microcapillary gas liquid chromatography as described before [2]. The fatty acids are expressed as a percentage from total phospholipids measured (C14/C22). The inter-assay coefficient of variation for determination of the different fatty acids by this method ranged between 2.6% and 9.1%, reflecting the high reproducibility of the assay.

### ***Assessment of apoptotic and proliferation indices by immunohistochemistry***

Sections of formalin-fixed, paraffin-embedded LAPC-4 tumor tissues (5 microns) were tested for the presence Ki-67 and caspase-3 [1:50], using an avidin biotin-complex technique and steam heat-induced antigen retrieval. Cells were defined as apoptotic if the whole nuclear area of the cell labeled positively for caspase-3. Apoptotic bodies were defined as small positively labeled globular bodies in the cytoplasm of the tumor cells (singly or in groups). To estimate the apoptotic index (the percentage of apoptotic events in a given area), apoptotic cells and bodies were counted in 10 high power fields and this figure were divided by the number of tumor cells in the same high-power fields. We also estimated the apoptotic index by light microscopy using hematoxylin stained slides from the same tumor sections as the Caspase-3. The intensity of staining in 10 high-power fields were scored descriptively or semi-quantitatively by pathologist as 1+: (0-25 % positive cells); 2+: (25-50 % positive cells); 3+: (50-75 % positive cells), and  $\geq 4+$ : (75-100 % positive cells) in a blinded manner. Proliferation was similarly examined and index estimated as total Ki-67 labeled cells / total cells counted.

### ***Statistical Analyses***

Statistical analyses (SAS ver 5.0) were performed by Student's t test or ANOVA. Correlations between outcome variables were computed using the Spearman correlation coefficient.  $P \leq 0.05$  was considered significant. Data are expressed as mean  $\pm$  standard deviation (SD).

## **KEY RESEARCH ACCOMPLISHMENTS IN YEARS 1 and 2:**

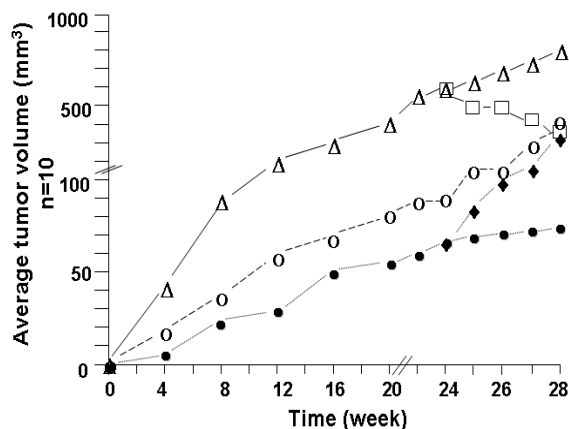
- (1) Wild type mice did not exhibit any prostate-specific phenotypic changes regardless of their diet.
- (2) Given that FLiMP<sup>+/+</sup> mice express 15-LO-1 and that these enzymes convert n-6 LA to the pro-tumorigenic metabolite, 13-HODE, as expected, FLiMP<sup>+/+</sup> mice fed a diet high LA diet exhibited more aggressive PIN, with PIN-like changes observed in as early as 10 weeks compared to FLiMP<sup>+/+</sup> mice fed a normal diet (PIN observed) and,
- (3) Our current study in year 2 provides mechanistic roles of omega ( $\omega$ )-3 fatty acids in slowing PCa growth by altering  $\omega$ -6/  $\omega$ -3 ratios via diet, and promoting apoptosis and inhibiting proliferation in tumors by directly competing with  $\omega$ -6 fatty acids for 15-LO-1, and COX-2 activities.

**REPORTABLE OUTCOMES:** Kelavkar U, Hutzley J, McHugh K, Allen KGD and Parwani A (2009). Prostate tumor growth can be modulated by dietarily targeting the 15-lipoxygenase (LO)-1 and cyclooxygenase (COX)-2 enzymes. Neoplasia, *Communicated*.

**CONCLUSION:** Omega ( $\omega$ )-3 fatty acids can slow PCa growth by altering  $\omega$ -6/  $\omega$ -3 ratios via diet, and promote apoptosis and inhibit proliferation in tumors by directly competing with  $\omega$ -6 fatty acids for 15-LO-1, and COX-2 enzyme activities.

## SUPPORTING DATA:

### Effect of diets on tumor growth



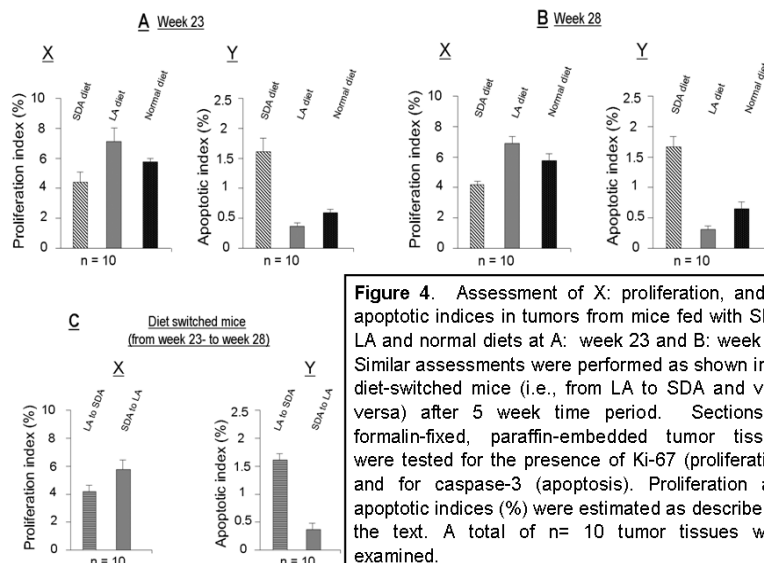
**Figure 1:** Average tumor volume per week in experimental mice. The tumor dimensions were measured using a caliper. Tumor volumes were calculated using the formula: length X width X height X 0.5236 (correction factor). For clarity, the standard deviation (SD) error bars from the mean of 10 values [SD was <10% of the mean] have been omitted. O-----O: Normal (control) diet fed mice;  $\Delta$ ----- $\Delta$ : LA ( $\omega$ -6) fed mice;  $\bullet$ ----- $\bullet$ : SDA ( $\omega$ -3) fed mice;  $\square$ ----- $\square$ : Switch from LA to SDA diet, and  $\blacklozenge$ ----- $\blacklozenge$ : Switch from SDA to LA diet respectively.

mice as compared with those from LA-fed mice (**Figure 2-shown as Figure 4**). We used gas chromatography to show that levels of LA, AA, EPA and DHA in membrane phospholipids of both red blood cells (**Table 1A**) and tumors (**Table 1B**) reflect these dietary changes. This result supports the use of RBC membranes as an accurate measurement of PUFA intake.

To confirm effects of diet *in vivo*, we used nude mice injected with prostate cancer LAPC-4 cells. As in [2], mice [38 per group] were fed high  $\omega$ -6 LA, high  $\omega$ -3 stearidonic acid (SDA, a precursor of EPA) or normal fat diets. After 23 weeks, 10 mice each from the LA and SDA diet groups were switched to the SDA and LA diets, respectively, and tumors were harvested 5 weeks later. Throughout the experiment, LA-fed mice had the highest mean tumor volume while SDA-fed mice had the lowest, which was altered by switching diets (**Figure 1**).

### Effect of diets on proliferation and apoptotic indices in tumors

Not surprisingly, immunohistochemistry revealed a decrease in the proliferation index (Ki-67 staining) and an increase in the apoptotic index (caspase 3 staining) in tumors from SDA-fed



**Figure 4.** Assessment of X: proliferation, and Y: apoptotic indices in tumors from mice fed with SDA, LA and normal diets at A: week 23 and B: week 28. Similar assessments were performed as shown in C: diet-switched mice (i.e., from LA to SDA and vice-versa) after 5 week time period. Sections of formalin-fixed, paraffin-embedded tumor tissues were tested for the presence of Ki-67 (proliferation) and for caspase-3 (apoptosis). Proliferation and apoptotic indices (%) were estimated as described in the text. A total of n= 10 tumor tissues were examined.

### Modulation of 15-LO-1, COX and key enzymes of the $\omega$ -6 pathway in tumors

In comparison to mice fed on normal diet, we observed a 2.7-fold increase in the  $\omega$ -6/  $\omega$ -3 ratio in tumors from LA-fed mice and a 4.2-fold decrease in the ratio in tumors from the SDA-fed mice (**Table 1C**). In view of the importance of PUFA metabolism in PCa, the increased estimated activity of  $\Delta$ -6-desaturase enzyme in tumors from mice fed the SDA-diet and the decreased activity in tumors from mice fed the LA-diet may be associated with the accumulation of the ant-proliferative  $\gamma$ -linolenic acid (GLA) and dihomo- $\gamma$ -linolenic acid (DGLA) [6]. Similarly, a reduced estimated  $\Delta$ -5-desaturase activity in the tumors from SDA diet cohort confirms the

previous observation from *in vitro* studies with LAPC-4 cells [2] suggesting that  $\omega$ -3 SDA inhibits  $\Delta$ -5-desaturase enzyme activity. This then further limits the formation of DHA as well as causes the accumulation of  $\gamma$ -linolenic acid and dihomogamma-linolenic acid. Interestingly, the estimated activity of the anabolic  $\omega$ -9 monounsaturated fatty acid pathway enzyme,  $\Delta$ -9-desaturase which is a member in the fatty acid synthase (FASN) pathway, increased in tumors from SDA-diet fed mice while decreased in tumors from LA-diet fed mice, suggesting that SDA favors the conversion of Stearic acid (18:0, n-9) to Oleic acid (18:1, n-9), this conversion otherwise is inhibited by LA. Together, these observations provide additional support for the role of dietary  $\omega$ -3 in slowing PCa growth.

#### A RBC's membrane analysis

Diet group n=10 % of total phospholipids	Normal		LA diet		SDA diet		$\omega$ -6 $\rightarrow\omega$ -3	$\omega$ -3 $\rightarrow\omega$ -6
	23 <sup>rd</sup> week	28 <sup>th</sup> week	23 <sup>rd</sup> week	28 <sup>th</sup> week	23 <sup>rd</sup> week	28 <sup>th</sup> week	5 <sup>th</sup> week	5 <sup>th</sup> week
Linoleic acid (LA)	6.2 ± 0.6	5.9 ± 0.8	12.2 ± 0.6	11.7 ± 0.6	2.2 ± 0.1	2.4 ± 0.1	3.2 ± 0.6	11.4 ± 0.3
Arachidonic acid (AA)	6.3 ± 0.3	6.7 ± 0.4	8.2 ± 0.6	8.4 ± 0.5	6.1 ± 0.1	6.4 ± 0.3	6.4 ± 0.6	7.1 ± 0.7
Eicosapentaenoic acid (EPA)	ND	ND	ND	ND	3.4 ± 0.2	3.2 ± 0.3	3.9 ± 1.2	0.9 ± 0.4
Docosahexaenoic acid (DHA)	ND	ND	ND	ND	6.2 ± 0.8	5.9 ± 1.2	5.8 ± 1.2	0.4 ± 0.06

#### B Tumor tissue membrane analysis

Diet group n=10 % of total phospholipids	Normal		LA diet		SDA diet		$\omega$ -6 $\rightarrow\omega$ -3	$\omega$ -3 $\rightarrow\omega$ -6
	23 <sup>rd</sup> week	28 <sup>th</sup> week	23 <sup>rd</sup> week	28 <sup>th</sup> week	23 <sup>rd</sup> week	28 <sup>th</sup> week	5 <sup>th</sup> week	5 <sup>th</sup> week
Linoleic acid (LA)	5.9 ± 0.4	5.8 ± 0.7	12.4 ± 0.4	12.1 ± 0.7	2.5 ± 0.7	2.3 ± 0.9	3.1 ± 0.5	11.8 ± 0.4
Arachidonic acid (AA)	6.1 ± 0.3	6.2 ± 0.3	7.8 ± 0.5*	7.9 ± 0.7	6.4 ± 0.2	6.1 ± 0.5	6.6 ± 0.8	6.9 ± 0.8
Eicosapentaenoic acid (EPA)	ND	ND	ND	ND	3.3 ± 0.3	3.5 ± 0.1	3.4 ± 0.5	1.1 ± 0.2
Docosahexaenoic acid (DHA)	ND	ND	ND	ND	6.4 ± 0.4	5.7 ± 0.6	5.9 ± 1.1	0.6 ± 0.04

#### C Phospholipid and enzyme activity analyses in tumors

Diet group at week 23	% Unsaturated of total phospholipids	% Saturated of total phospholipids	PUFA n-6/n-3	Elongase	Delta -5 desaturase	Delta -6 desaturase	Delta -9 desaturase
Normal	49.4 ± 1.7	42.7 ± 1.5	3.4 ± 1.1	84.1 ± 1.7	7.1 ± 0.6	3.1 ± 0.6	12.7 ± 0.6
LA diet	51.1 ± 1.2	44.1 ± 1.3	9.4 ± 1.1	110.1 ± 1.6	14.4 ± 0.3	7.4 ± 0.5	7.1 ± 0.7
SDA diet	50.2 ± 0.9	43.2 ± 1.1	0.8 ± 0.1	42.2 ± 0.9	0.78 ± 0.06	12.9 ± 1.2	28.7 ± 1.2
Diet group at week 28	% Unsaturated of total phospholipids	% Saturated of total phospholipids	PUFA n-6/n-3	Elongase	Delta -5 desaturase	Delta -6 desaturase	Delta -9 desaturase
Normal	48.7 ± 1.1	43.2 ± 1.7	3.5 ± 1.3	86.1 ± 2.4	6.9 ± 0.8	3.3 ± 0.2	13.0 ± 0.5
LA [High fat, n-6]	52.3 ± 1.1	43.7 ± 1.4	9.2 ± 1.3	115.3 ± 2.3	16 ± 0.5	6.2 ± 0.7	6.2 ± 0.7
LA to SDA (after 5 weeks exposure)	50.1 ± 1.3	42.8 ± 1.3	2.4 ± 0.6	32.3 ± 0.7	0.92 ± 0.04	15.4 ± 2.2	25.4 ± 2.6
SDA diet	49.5 ± 1.4	44.1 ± 1.5	0.9 ± 0.2	37.4 ± 1.7	0.59 ± 0.05	11.7 ± 1.7	27.7 ± 1.7
SDA to LA (after 5 weeks of exposure)	52.6 ± 0.7	43.3 ± 1.1	8.1 ± 0.4	108.1 ± 2.8	12.2 ± 0.01	6.8 ± 0.2	7.2 ± 1.2

#### D 15-LO-1 and COX enzyme activity analyses

Metabolic products	13-HODE	15-HETE	15-HEPE	Total prostaglandins
Diet group at week 23 (n=10)				
Normal	0.9 ± 0.2	0.1**	0	6.1 ± 0.33
LA diet	2.1 ± 0.2	ND	0	6.2 ± 0.46
SDA diet	ND	†	1.8 ± 0.06	7.1 ± 0.26
LA to SDA (after 5 weeks time point)	0.2 ± 0.1	ND	1.4 ± 0.03	7.6 ± 0.54
SDA to LA (after 5 weeks time point)	1.8*	ND	0.3 ± 0.03	6.4 ± 0.22

**Table 1:** Mice were fed with normal, LA and SDA diets. Composition of LA, AA, EPA and DHA in phospholipids (n=10) were determined. Fatty acid methyl esters were analyzed by gas liquid chromatography as described <sup>Φ</sup>. The fatty acids are expressed as percentage (%) from total phospholipids and represent the mean ± SD of 10 determinants.

**A:** RBC's membrane analysis,

**B:** Tumor tissue membrane analysis,

**C:** Phospholipid analysis of unsaturated and saturated fatty acids (PUFAs), PUFA ratios and activities of key enzymes involved in fatty acid biosynthesis. Fatty acid methyl esters were analyzed by gas chromatography and enzyme activities were estimated as the product-to-precursor ratios of the percentages of individual fatty acids as described <sup>Φ</sup>. Values represent mean ± SD of 10 determinations, and

**D:** Enzymes (15-LO-1 and COX) activity profiles from the tumors of mice fed with LA, SDA, LA to SDA and SDA to LA diets were measured as described <sup>Φ</sup>. Percent (%) conversion of the products represents mean ± SD of 10 determinations from one experiment. ND: Not detectable, \*: Observed in 1 tissue and, \*\*: Observed in 2 tissues, †: p<0.05 and ††: p<0.02. <sup>Φ</sup>: Kelavkar, *et al.*, (2006). *Neoplasia*, 8, 112-124.

As shown in **Table1D**, tumor lysate's from LA diet fed mice showed 2-fold higher levels of 13(S)-HODE versus normal diet fed mice and remained undetectable in SDA diet fed mice. Interestingly, when cohorts of mice were switched from  $\omega$ -6 diet to  $\omega$ -3 diet (from LA diet to SDA diet) there was a significant decrease in 13-HODE from 2.1 ± 0.2 to 0.2 ± 0.1 (n=10, p<0.05) and a decrease from 1.8 ± 0.06 to 0.3 ± 0.03 (n=10, p<0.02) in 15-HEPE when cohorts of mice were switched from  $\omega$ -3 diet to  $\omega$ -6 diet (from SDA diet to LA diet). This confirmed 15-LO-1 activity in the tumors. Although, we did not observe major differences in the total prostaglandin levels in either diet fed or diet switch groups, formation of prostaglandins confirmed COX activity. The absence of detectable 15-HETE indicated either a deficiency in 15-LO-2 enzyme or poor 15-LO-1/AA metabolism. Importantly, these results further corroborate our previous *in vitro* and *in vivo* studies that SDA (and EPA) do not inhibit the activities of either 15-LO-1 or COX and the tumor growth is modulated by the substrate competition of  $\omega$ -3 for 15-LO-1 enzyme with  $\omega$ -6 fatty acids [2].

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**APPENDICES:** Manuscript attached as a pdf file.

**Prostate tumor growth can be modulated by dietarily targeting the 15-lipoxygenase (LO)-1 and cyclooxygenase (COX)-2 enzymes**

Kelavkar Uddhav<sup>1</sup>, Hutzley Justin<sup>2</sup>, McHugh Kevin<sup>2</sup>, Allen Kenneth GD<sup>3</sup> and Parwani Anil<sup>4</sup>

<sup>1</sup>Division of Hematology/Oncology and Cancer Institute, <sup>2</sup>Department of Urology, <sup>4</sup>Department of Pathology, University of Pittsburgh, Pittsburgh, PA, U.S.A and <sup>3</sup>Dept. Food Science and Human Nutrition, Colorado State University, Fort Collins, CO, USA.

<sup>1</sup>To whom correspondence should be addressed: Uddhav P. Kelavkar, Ph.D., G.12.E Hillman Cancer Center UPCI Research Pavilion, 5117 Centre Avenue, Pittsburgh, PA 15213-1863, USA. Tel: (412)-623-1110; Fax: (412)-623-4747 (e-mail: kelavkarup@upmc.edu).

**Key words:**

Polyunsaturated fatty acids, cancer prevention, omega ( $\omega$ ) diet, 15-lipoxygenase-1, cyclooxygenase-2, growth, apoptosis, prostate cancer

**Running title:** Dietarily targeting 15-LO-1 and COX-2 in prostate cancer

**Abbreviations:** 15-Lipoxygenase-1 (15-LO-1); Arachidonic acid (AA); Cyclooxygenase (COX); High Performance Liquid Chromatography (HPLC); Hydroxyeicosatetraenoic acid (HETE); Hydroxyoctadecadienoic acid (HODE); Lipoxygenase (LO); Linoleic acid (LA); prostaglandins (PGs); Los Angeles Prostate Cancer-4 (LAPC-4); polyunsaturated fatty acids (PUFAs).

## Abstract

The main objectives of our study were to determine the bioavailability of omega-( $\omega$ )-3 to the tumor, understand mechanisms, and the feasibility of targeting the  $\omega$ -6 polyunsaturated fatty acids (PUFAs) metabolizing 15-lipoxygenase (LO)-1 and cyclooxygenase (COX)-2 pathways. Nude mice injected subcutaneously (s.c) with LAPC-4 prostate cancer (PCa) cells were randomly divided into three different isocaloric (and same percent [%] of total fat) diet groups: high  $\omega$ -6 linoleic acid (LA), high  $\omega$ -3 stearidonic acid (SDA) PUFAs, and normal (control) diets. Tumor growth and apoptosis were examined as end-points after administration of a short-term (5-week)  $\omega$ -3 and  $\omega$ -6 fatty acid diets. Tumors were examined for growth, lipids, enzyme activities, apoptosis and proliferation. Tumors from the LA-diet fed mice exhibited the most rapid growth as compared with tumors from the control and SDA diet fed mice. Moreover, a diet switch from LA to SDA caused a dramatic decrease in the growth of tumors in 5 weeks whereas tumors grew more aggressively when mice were switched from a SDA to LA diet. Evaluating tumor proliferation (Ki-67) and apoptosis (Caspase-3) in mice fed the LA- and SDA-diets suggested increased % proliferation index from the  $\omega$ -6 diet fed mice as compared with tumors from the  $\omega$ -3 SDA-fed mice. Further, increased apoptosis was observed in tumors from  $\omega$ -3 SDA-fed mice versus tumors from  $\omega$ -6 diet fed mice. Levels of membrane phospholipids of red blood cells (RBCs) reflected dietary changes and correlated with the levels observed in tumors. LA or AA and metabolites (eicosanoid/prostaglandins) were analyzed for 15-LO-1 and COX-2 activities by high performance liquid chromatography (HPLC). We also examined the percent (%) unsaturated or % saturated fatty acids in the total phospholipids, PUFA  $\omega$ -6/  $\omega$ -3 ratios and other major enzymes (elongase, delta ( $\Delta$ )-5-desaturase, and  $\Delta$ -6-desaturase) of  $\omega$ -6 catabolic pathways from the tumors. We observed a 2.7-fold increase in the  $\omega$ -6/ $\omega$ -3 ratio in tumors from LA-fed mice and a 4.2-fold decrease in the ratio in tumors from the SDA-fed mice. There was an increased  $\Delta$ -6-desaturase and  $\Delta$ -9 desaturase enzyme activities and reduced estimated  $\Delta$ -5-desaturase activity in tumors from mice fed the SDA-diet. Opposite effects were observed in tumors from mice fed the LA-diet. Together, these observations provide mechanistic roles of  $\omega$ -3 fatty acids in slowing PCa growth by altering  $\omega$ -

6/  $\omega$ -3 ratios via diet, and promoting apoptosis and inhibiting proliferation in tumors by directly competing with  $\omega$ -6 fatty acids for 15-LO-1, and COX-2 activities.

## Introduction

Prostate cancer (PCa) still remains one of the leading causes of cancer death among men in the United States (US) [1]. Current therapies for PCa include watchful waiting, radical prostatectomy, hormonal therapy and targeted radiation. Unfortunately, all available therapies have associated risks and limitations and new therapeutic strategies are critically needed [2,3]. One promising strategy involves the use of dietary interventions [4,5]. International incidence patterns and migrations studies, epidemiological data as well as animal and *in vitro* studies indicate that consuming a diet rich in fat increases the risk for developing PCa [6-8]. Dietary fat also includes  $\omega$ -3 and  $\omega$ -6 polyunsaturated fatty acids (PUFAs), both of which play important roles in many human biological processes including prostate cancer [9,10]. As humans cannot synthesize  $\omega$ -3 and  $\omega$ -6 PUFAs, they are considered essential fatty acids. While all mammalian cells can interconvert the PUFAs within each series by elongation, desaturation and retro conversion, the two series are not interchangeable due to the lack of the *Fat-1* gene [11], which encodes the  $\omega$ -3 desaturase enzyme[12].

Linoleic acid (LA; 18:2 $\omega$ -6) represents a  $\omega$ -6 PUFA commonly found in high-fat Western diets [13]. Terrestrial plants synthesize LA, and once ingested by mammals, LA is either metabolized to 13-(S)-hydroxyoctadecadienoic acid [13-(S)-HODE] or converted further to arachidonic acid (AA; 20:4 $\omega$ -6). Three fatty acids, found primarily in fish oils, comprise the  $\omega$ -3 family: Alpha linolenic acid (ALA or  $\alpha$ -LNA; 18:3 $\omega$ -3), eicosapentaenoic acid (EPA; 20:5 $\omega$ -3), and docosahexaenoic acid (DHA; 22:6 $\omega$ -3). ALA, synthesized by cold-water vegetation, is converted by fish to EPA and DHA. Of note, through the same series of enzymes used to convert LA to AA, humans can synthesize EPA and DHA from ingested ALA. The conversion of dietary ALA to EPA and DHA is also dependent on the dietary  $\omega$ -6/ $\omega$ -3 ratio [13]. Importantly, US diets provide excessive LA [14] that competes with ALA for the desaturase and elongase enzymes, impeding the formation of EPA and DHA. Studies have suggested that the high incidence of PCa in Americans may, in part, result from an imbalance in the

ratio of  $\omega$ -3 to  $\omega$ -6 fatty acids because the typical American diet is low in  $\omega$ -3 and high in  $\omega$ -6 [9]. Recently, we demonstrated increased expression of a  $\omega$ -6 LA-metabolizing enzyme, 15-lipoxygenase-1 (15-LO-1, ALOX15) in prostate tumor tissues as compared with normal adjacent tissue [15-17]. Although AA can also act as a substrate for 15-LO-1, yielding the anti-inflammatory and pro-apoptotic metabolite 15-(S)-hydroxyeicosatetraenoic acid [15-(S)-HETE], the 15-LO-1 enzyme greatly prefers LA. Fifteen-LO-1 metabolizes LA to 13-(S)-HODE, which can regulate cell growth, differentiation and vascular homeostasis [15-17].

In addition, AA also acts as a substrate for cyclooxygenases (COX)-1 and -2 [18]. Most tissues constitutively express low protein levels of COX-1 and no COX-2. Growth factors or inflammatory agents rapidly induce COX-2 expression in prostate (35) and studies show overexpression of COX-2 in PCa [19,20]. COX-2 overexpression, leading to the production of pro-inflammatory prostaglandins (e.g., PGE<sub>2</sub>), possibly contributes to PCa pathobiology [21]. Recently, a study has shown that the combination of DHA and celecoxib (COX-2 specific inhibitor) prevents prostate cancer cell growth *in vitro* [22]. This inhibition of disease development likely results, in part, from the ability of the  $\omega$ -3 PUFA, EPA, to successfully compete with LA and AA for 15-LO-1 and COX-2 respectively. 15-LO-1 metabolizes EPA to 15-hydroxyeicosapentaenoic acid [15-HEPE] [23], a metabolite shown to have anti-tumorigenic properties, whereas COX-2 metabolizes EPA to the anti-inflammatory as well as anti-tumorigenic prostaglandin PGE<sub>3</sub> [23,24]. The  $\omega$ -3 PUFA eicosapentaenoic acid (EPA) also serves as a substrate for 15-LO-1 and COX-2, but metabolism of EPA by these enzymes results in the formation of anti-tumorigenic products. Therefore,  $\omega$ -3 fatty acids may not only decrease production of the pro-tumorigenic metabolites derived from the  $\omega$ -6 fatty acid pathway, but may also result in increased production of anti-tumorigenic metabolites.

Based on these and our previous observation [25], we further hypothesized that prostate tumor growth can be modulated by dietarily targeting the 15-LO-1 and cyclooxygenase (COX)-2 enzymes. This manuscript describes experiments designed to show that dietarily targeting the 15-LO-1 and cyclooxygenase (COX)-2 enzymes *in vivo*, can slow PCa progression.

## **Materials and Methods**

### ***Cell Culture***

Los Angeles Prostate Cancer-4 (LAPC-4) PCa cells were kindly provided by Dr. Robert Reiter (University of California-Los Angeles) and maintained in phenol red-free DMEM (Invitrogen, Carlsbad, CA) containing 5% heat-inactivated fetal calf serum (FCS, Sigma) with streptomycin-penicillin antibiotics in a 5% CO<sub>2</sub> incubator at 37°C.

### ***15-LO-1 and COX enzyme activities in tumors***

The activities of 15-LO-1 and COX were estimated as described previously by us [26] and expressed as percent conversion of substrate/s to product/s.

### ***Enzyme activity calculation of other enzymes of eicosanoids pathway***

The activities of enzymes involved in fatty acid biosynthesis were estimated as the product-to-precursor ratios of the percentages of individual fatty acids [27]. The estimated enzyme activities included those of elongase, calculated as the stearic acid (18:0)-to-palmitic acid (16:0) ratio; delta ( $\Delta$ )-5 desaturase, calculated as the arachidonic acid [20:4( $\omega$ -6)]-to-di-homo-gamma ( $\gamma$ )-linolenic acid [20:3( $\omega$ -6)] ratio;  $\Delta$ -6 desaturase, calculated as the di-homo- $\gamma$ -linolenic acid [20:3( $\omega$ -6)]-to-linoleic acid [18:2( $\omega$ -6)] ratio (assuming that  $\Delta$ -6 desaturase and not elongase is rate limiting); and  $\Delta$ -9 desaturase, calculated as the oleic acid [18:1( $\omega$ -9)]-to-stearic acid [18:0 ( $\omega$ -9)] ratio.

### ***Diets, experimental strategy, feeding Protocol, LAPC-4 cell injection and tumor analyses***

We utilized three different isocaloric diets (caloric density 4.4 kcal/gm) for our study [Table 1]. These custom-made, semi-purified diets were prepared and irradiated by Purina Test Diet, Inc. (Richmond, IN). High  $\omega$ -6 LA and high  $\omega$ -3 SDA fat diet group (experimental) were identical to those that are described before [26]. Use of pure EPA is cost-prohibitive and therefore SDA, an immediate



precursor of EPA, served as a legitimate substitute. Furthermore, because of concerns with mercury and polychlorinated biphenyl contamination of fish and fish-oils, which currently provide the major sources of long-chain  $\omega$ -3 in the human diet, land based sources of functional  $\omega$ -3 fatty acids such as SDA are currently being developed [28]. Normal-fat diet (control) was also isocaloric when adjusted by adding dextrin and corn-oil.

The experimental strategy is illustrated in detail in Figure 2. Initially we took a total of 101 athymic male BALB/C nude (nu/nu) (6–8 weeks old) mice obtained from Charles River (Wilmington, MA) and fed them initially a no fat diet for 2 weeks as described before [26]. As illustrated in Figure 2, after 2 weeks, we split these mice into 3 groups namely; two experimental groups [a total of n=76 divided into 38 mice per high  $\omega$ -6 LA and  $\omega$ -3 SDA fat diet groups (experimental)] versus the control group (n=25 per normal dietary group). These mice were housed in single sterile animal cages to allow for the maintenance of isocaloric intake between the diet groups. Cages, bedding, and water were autoclaved before use. Specially designed feeding receptacles were placed in the cages so that food intake could be carefully monitored. Sterile techniques were used whenever handling the cages, mice, and food. The Pittsburgh Animal Research Committee approved the experiments, and animals were cared for in accordance with institutional guidelines.

After 2 weeks on no fat diet, all the n=101 mice were injected with LAPC-4 cells. This time point was counted as week 1. The mice were injected in duplicate subcutaneously (s.c.) in the right and left lateral flank with  $1 \times 10^6$  LAPC-4 tumor cells in 0.1 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA). When tumors became palpable, the tumor dimensions were measured [29]. Tumor growth and apoptosis were examined as end-points after administration of different experimental and control short-term (5-week) diets. At week 23, half of the mice from each PUFA group were switched to the opposing diets (i.e., from SDA to LA diet and vice versa) while the other half of the mice

remained on their original diet. Tumors were examined for growth, lipids, enzyme activities, apoptosis and proliferation indices.

#### ***Red blood cells membrane (RBC's) and tumor phospholipid content analyses***

Phospholipid content from RBC's and tumor tissues membranes were analyzed by temperature-programmed microcapillary gas liquid chromatography as described before [26]. The fatty acids are expressed as a percentage from total phospholipids measured (C14/C22). The inter-assay coefficient of variation for determination of the different fatty acids by this method ranged between 2.6% and 9.1%, reflecting the high reproducibility of the assay.

#### ***Assessment of apoptotic and proliferation indices by immunohistochemistry***

Sections of formalin-fixed, paraffin-embedded LAPC-4 tumor tissues (5 microns) were tested for the presence Ki-67 and caspase-3 [1:50], using an avidin biotin-complex technique and steam heat-induced antigen retrieval. Cells were defined as apoptotic if the whole nuclear area of the cell labeled positively for caspase-3. Apoptotic bodies were defined as small positively labeled globular bodies in the cytoplasm of the tumor cells (singly or in groups). To estimate the apoptotic index (the percentage of apoptotic events in a given area), apoptotic cells and bodies were counted in 10 high power fields and this figure were divided by the number of tumor cells in the same high-power fields. We also estimated the apoptotic index by light microscopy using hematoxylin stained slides from the same tumor sections as the Caspase-3. The intensity of staining in 10 high-power fields were scored descriptively or semi-quantitatively by pathologist as 1+: (0-25 % positive cells); 2+: (25-50 % positive cells); 3+: (50-75 % positive cells), and  $\geq 4+$ : (75-100 % positive cells) in a blinded manner. Proliferation was similarly examined and index estimated as total Ki-67 labeled cells / total cells counted.

### ***Statistical Analyses***

Statistical analyses (SAS ver 5.0) were performed by Student's t test or ANOVA. Correlations between outcome variables were computed using the Spearman correlation coefficient.  $P \leq 0.05$  was considered significant. Data are expressed as mean  $\pm$  standard deviation (SD).

## Results

### *Effect of diets on tumor growth*

We initially examined the effects of the specific diets (i.e., normal,  $\omega$ -3 SDA and  $\omega$ -6 LA) on tumor growth (n=20 mice/diet group) over a total of 28 week time period (Table 1 and Figure 1). At week 23, ten mice from  $\omega$ -3 SDA diet fed mice were switched to  $\omega$ -6 LA diet and ten mice from  $\omega$ -6 LA diet fed mice were switched to  $\omega$ -3 SDA diet. The remaining 10 mice continued to be fed on their original diets. Twenty normal diet fed mice served as controls. Tumors from each of these cohorts were measured/week for 5 more weeks (i.e., from week 24-28). Tumors from the LA-diet fed mice exhibited the most rapid growth as compared with tumors from the control and SDA diet fed mice. Moreover, a diet switch from LA to SDA caused a dramatic decrease in the growth of tumors in 5 weeks (from week 23 to 28) whereas tumors grew more aggressively when mice were switched from a SDA to LA diet in the same time period (Figure 1).

### *Effect of diets on proliferation and apoptotic indices in tumors*

Based on the experimental strategy outlined in Figure 2, we employed immunohistochemistry to evaluate tumor proliferation (Ki-67) and apoptosis's (Caspase-3) in mice (n=10/diet groups) fed the normal, LA- and SDA-diets at week 23 (Figure 3A) and week 28 (Figure 3B) time points. As depicted in Figures 3AX and 3BX, calculated % proliferation index was between 4-5 $\pm$ 0.2-1 in SDA diet fed mice, 7 $\pm$ 1-1.5 in LA diet fed mice and 5.8 $\pm$ 0.3-0.4 in normal diet fed mice respectively. Simultaneously, as depicted in Figures 3AY and 3BY, calculated % apoptotic index was between 1.6-1.7 $\pm$ 0.2-0.3 in SDA diet fed mice, 0.4 $\pm$ 0.1 in LA diet fed mice and 0.6 $\pm$ 0.2-0.1 in normal diet fed mice respectively. As expected, there were no apparent major differences in week 28 versus week 23 in either proliferation or apoptosis indices. However, as shown in Figures 3CX, we assessed proliferation, and 3CY: apoptotic indices in tumors after 5 week time period (from week 24 to week 28) from diet-switched mice (i.e., from LA to SDA and vice-versa, n=10/diet group). We observed a dramatic decrease in the proliferation index and an increase in apoptotic index in tumors from mice

that were switched from LA to SDA diet. Interestingly, these changes were reversed in tumors from mice that were switched from SDA to LA diet.

### ***RBC's and tumor phospholipid content analyses***

As shown in Table 2A (RBC's) and 2B (tumors), levels of  $\omega$ -6 LA, AA and  $\omega$ -3 EPA and DHA in membrane phospholipids of RBC's reflected dietary changes in normal, LA and SDA diet fed mice (n=10/diet groups). There were no significant differences in membrane phospholipid content in both these tissues observed at week 23 versus week 28 (a 5 week time period). This study also ascertained that the red blood cells membrane phospholipid analysis is suitable for accurate measurement of dietary intake of PUFAs. Interestingly, when cohorts of mice (n=10) were switched from  $\omega$ -6 diet to  $\omega$ -3 diet and vice-versa and allowed to feed further for a 5-week time period, the levels of the  $\omega$ -6 and  $\omega$ -3 PUFAs in both; RBC's and tumor membrane phospholipids, were also modulated accordingly (Table 2A and 2B).

### ***Modulation of 15-LO-1, COX and key enzymes of the $\omega$ -6 pathway in tumors***

To assess whether or not the tumor modulating effect of  $\omega$ -3 SDA (anti-tumorigenic),  $\omega$ -6 LA (pro-tumorigenic) diets and suggestive of their actions in diet switching experiments, we examined tissue metabolites from different dietary cohorts by HPLC analysis (Table 3). Tumor lysate's from LA diet fed mice showed 2-fold higher levels of 13(S)-HODE versus normal diet fed mice and remained undetectable in SDA diet fed mice. Interestingly, when cohorts of mice were switched from  $\omega$ -6 diet to  $\omega$ -3 diet (from LA diet to SDA diet) there was a significant decrease in 13-HODE from  $2.1 \pm 0.2$  to  $0.2 \pm 0.1$  (n=10, p<0.05) and a decrease from  $1.8 \pm 0.06$  to  $0.3 \pm 0.03$  (n=10, p<0.02) in 15-HEPE when cohorts of mice were switched from  $\omega$ -3 diet to  $\omega$ -6 diet (from SDA diet to LA diet). This confirmed 15-LO-1 activity in the tumors. Although, we did not observe major differences in the total prostaglandin levels in either diet fed or diet switch groups, formation of prostaglandins confirmed

COX activity. We did not find detectable levels of 15-HETE indicated either a deficiency in 15-LO-2 enzyme or poor 15-LO-1/AA metabolism.

In comparison with mice fed the normal diet, we observed a 2.7-fold increase in the  $\omega$ -6/  $\omega$ -3 ratio in tumors from LA-fed mice and a 4.2-fold decrease in the ratio in tumors from the SDA-fed mice (Table 4). In view of the importance of PUFA metabolism in PCa, the increased estimated activity of  $\Delta$ -6-desaturase enzyme in tumors from mice fed the SDA-diet and the decreased activity in tumors from mice fed the LA-diet. Similarly, we observed a reduced estimated  $\Delta$ -5-desaturase activity in the tumors from SDA diet cohort. Interestingly, the estimated activity of the anabolic n-9 monounsaturated fatty acid pathway enzyme,  $\Delta$ -9-desaturase, increased in tumors from SDA-diet fed mice while decreased in tumors from LA-diet fed mice (Table 4).

## Discussion:

Ideally, the ratio of  $\omega$ -3 to  $\omega$ -6 PUFAs in the human body should be 1:4 to 1:1 (71). Typical US diet is low in  $\omega$ -3 and high in  $\omega$ -6 and many individuals contain 10 to 20 times more  $\omega$ -6 PUFAs than  $\omega$ -3 PUFAs (72-74) and international incidence patterns and migrations studies, epidemiological data as well as animal and *in vitro* studies indicate that consuming a diet rich in fat increases the risk for developing PCa [30], [8], [31-34].

Our previous *in vitro* and *in vivo* studies demonstrate the presence of  $\omega$ -6 Linoleic acid (LA)-metabolizing enzyme 15-LO-1 at higher levels in PCa and as a key enzyme that contributes to the initiation and development of the neoplastic phenotype in PCa [16,17,26,29,35-37]. In this study we provide information on the mechanisms of how the metabolites of anti-tumorigenic  $\omega$ -3 fatty acids can modulate the protumorigenic  $\omega$ -6 LA and AA enzymatic pathways.

Our hypothesis was that diet high in  $\omega$ -3 PUFAs competes with  $\omega$ -6 LA and –AA as a substrate for the enzyme 15-LO-1, upregulated in PCa, resulting in reduced levels of the pro-tumorigenic metabolite of LA, 13(S)-HODE, and increased levels of the anti-tumorigenic metabolites EPA (SDA metabolite), 15-HEPE and PGE<sub>3</sub>. EPA can also compete with AA as a substrate for the COX-2 enzyme, also upregulated in PCa, resulting in reduced levels of the pro-inflammatory metabolite of AA, PGE<sub>2</sub>, and increased levels of the anti-inflammatory metabolites of EPA, PGE<sub>3</sub>. The absence of detectable 15-HETE indicated either a deficiency in 15-LO-2 enzyme or poor 15-LO-1/AA metabolism [38-40]. Importantly, these results further corroborate our previous *in vitro* and *in vivo* study that SDA (and EPA) do not inhibit the activities of either 15-LO-1 or COX and tumor growth reflects the substrate competition of  $\omega$ -3 with  $\omega$ -6 fatty acids [25].

The increased estimated activity of  $\Delta$ -6-desaturase enzyme in tumors from mice fed the SDA-diet and the decreased activity in tumors from mice fed the LA-diet could be associated with the accumulation of the ant-proliferative  $\gamma$ -linolenic acid (GLA) and dihomo- $\gamma$ -linolenic acid (DGLA) [41]. Similarly, reduced estimated  $\Delta$ -5-desaturase activity in the tumors from SDA diet cohort confirms the previous observation from *in vitro* studies with LAPC-4 cells [25], suggesting that  $\omega$ -3 SDA inhibits  $\Delta$ -5-desaturase enzyme activity. This then further limits the formation of DHA as well as causes the accumulation of  $\gamma$ -linolenic acid and dihomo- $\gamma$ -linolenic acid.

The increased estimated activity of the anabolic  $\omega$ -9 monounsaturated fatty acid pathway enzyme,  $\Delta$ -9-desaturase, in tumors from SDA-diet fed mice but decrease activity in LA-diet fed mice suggests that SDA can favor the conversion of Stearic acid (18:0, n-9) to Oleic acid (18:1, n-9). However, this conversion is inhibited by LA. Together, these observations provide additional support for the role of  $\omega$ -3 SDA in slowing PCa growth. The observation of  $\Delta$ -9-desaturase activity modulation by SDA may also suggest that the tumor inhibition is either independent of oleic acid action [42]. This is particularly an important consideration for clinical trial studies in PCa patients using oleic acid as a placebo.

In addition to 15-LO-1, evidence also suggests that other metabolic enzymes play important roles in PCa pathobiology. Arachidonic acid obtained via diet or LA metabolic conversion, is the preferred substrate for 15-lipoxygenase (LO)-2. Fifteen-LO-2 converts AA to 15-(S)-HETE, a metabolite shown to both enhance apoptosis and act as a negative cell cycle regulator [43]. Furthermore, 15-(S)-HETE has anti-inflammatory properties [24,44,45] and studies support an association between inflammation and PCa [46], [47]. Similarly, AA also acts as a substrate for cyclooxygenase (COX)-2 leading to the production of prostaglandins (PG), such as PGE<sub>2</sub>, that have pro-inflammatory effects and thus possibly contribute to PCa pathobiology. Although the present study did not specifically evaluate for both COX-1 and -2 levels or for prostaglandins such as PGE<sub>2</sub> versus PGE<sub>3</sub>, previous studies have



demonstrated overexpression of COX-2 [19], [20] and an anti-tumorigenic role of PGE<sub>3</sub> in PCa [48], [23]. Therefore, due to the abundance of COX-2 in PCa tissue, it was reasonable to assume that the vast majority of AA in tumors cells would be converted to PGE<sub>2</sub> if ω-3s were low or absent.

More specifically, use of dietary ω-3 PUFAs as agents for cancer prevention can prove a valuable strategy in the fight against PCa (53-62) and recurrence. Consequently 15-LO-1 metabolizes EPA to 15-hydroxyeicosapentaenoic acid [15-HEPE] (39), a metabolite shown to have anti-tumorigenic properties, whereas COX-2 that metabolizes EPA to the anti-inflammatory as well as anti-tumorigenic prostaglandin PGE<sub>3</sub> (39-41). Therefore, based on the substrates i.e., dietary ω-6 or ω-3 in concert with the activities of 15-LO-1 and COXs, cells can be predisposed to either a proliferative or anti-proliferative outcome. This effect seemed to be independent of percent (%) unsaturated or % saturated fatty acids in the total phospholipids as observed in tumors and suggests that the cells are able to maintain membrane fluidity and functionality of proteins in the membrane.

This study supported our hypothesis and explains the observation that, 1) EPA and LA both compete as substrates for the enzyme 15-LO-1, which results in a decrease in the pro-tumorigenic metabolite of LA, 13(S)-HODE, and an increase in the anti-tumorigenic metabolite of EPA, 15-HEPE, and 2) EPA and AA both compete as substrates for the enzyme COX-2, which may result in production of “good” prostaglandins (for e.g., PGE<sub>3</sub>). Thus, SDA derived EPA alone, EPA derived fatty acids such as DHA or a combination in fish-oil are promising dietary intervention agents against PCa aimed at 15-LO-1 and COX-2 as the molecular targets.

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**Legends to figures:**

**Figure 1:** Average tumor volume per week in experimental mice. The tumor dimensions were measured using a caliper. Tumor volumes were calculated using the formula: length X width X height X 0.5236 (correction factor). For clarity, the standard deviation (SD) error bars from the mean of 10 values [SD was <10% of the mean] have been omitted. O-----O: Normal (control) diet fed mice; Δ-----Δ: LA (ω-6) fed mice; ●-----●: SDA (ω-3) fed mice; □-----□: Switch from LA to SDA diet, and ♦-----♦: Switch from SDA to LA diet respectively.

**Figure 2:** Experimental strategy.

**Figure 3:** Assessment in A: week 23, and B: week 28 time period of X: proliferation, and Y: apoptotic indices in tumors from mice fed with SDA (▨), LA (▩) and normal diets (▤). Similar assessments were performed as shown in C: diet-switched mice [i.e., from LA to SDA (▨▩) and vice-versa (▩▨)] after 5 week time period. Sections of formalin-fixed, paraffin-embedded tumor tissues were tested for the presence of Ki-67 (proliferation marker) and for caspase-3 (apoptosis marker). Proliferation and apoptotic indices (%) were estimated as described in the Material and Methods section. A total of n= 10 tumor tissues/group were examined.

**Table 1:** Composition of isocaloric diets (gm/100 gm). Caloric density is 4.4 kcal/gm. †: Values provided by the supplier.

**Table 2:** Composition of LA, AA, EPA and DHA in phospholipids (n=10). A: RBC membranes, and B: Tumors from mice fed with normal, LA and SDA diets. Fatty acid methyl esters were analyzed by gas liquid chromatography as described in the Materials and Methods section. The fatty acids are expressed as percentage (%) from total phospholipids and represent the mean ± SD of 10 determinants.

**Table 3:** Enzymes (15-LO-1 and COX) activity profiles from the tumors of mice fed with LA, SDA, LA to SDA and SDA to LA diets were measured as described in the Materials and Methods section. Percent (%) conversion of the products represents mean  $\pm$  SD of 10 determinations from one experiment. ND: Not detectable, \*: Observed in 1 tissue and, \*\*: Observed in 2 tissues, †:  $p < 0.05$  and ††:  $p < 0.02$ .

**Table 4:** Phospholipid analysis of unsaturated and saturated fatty acids (PUFAs), PUFA ratios and activities of key enzymes involved in fatty acid biosynthesis. Fatty acid methyl esters were analyzed by gas chromatography and enzyme activities were estimated as the product-to-precursor ratios of the percentages of individual fatty acids as described in the Materials and Methods section. Values represent mean  $\pm$  SD of 10 determinations.

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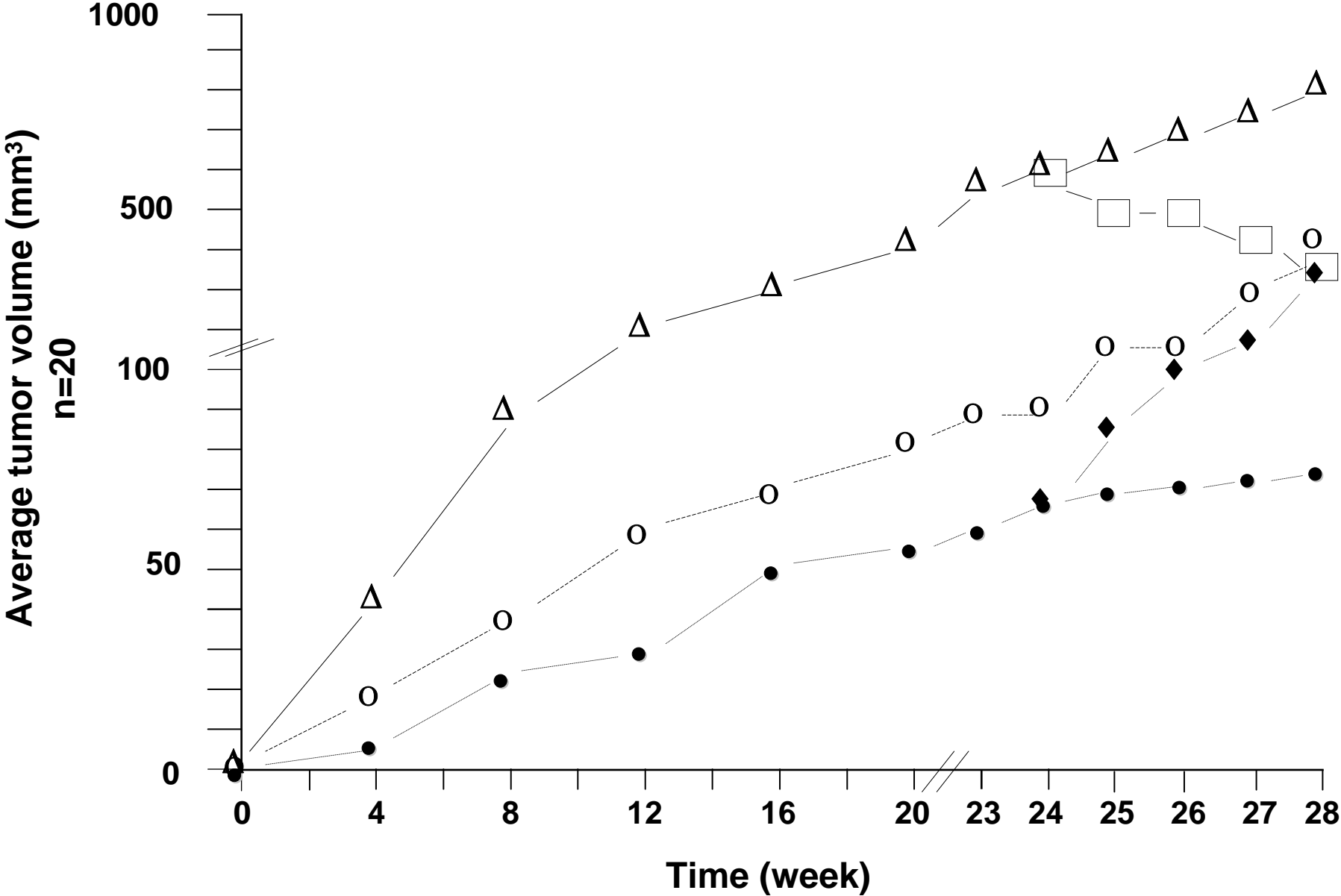
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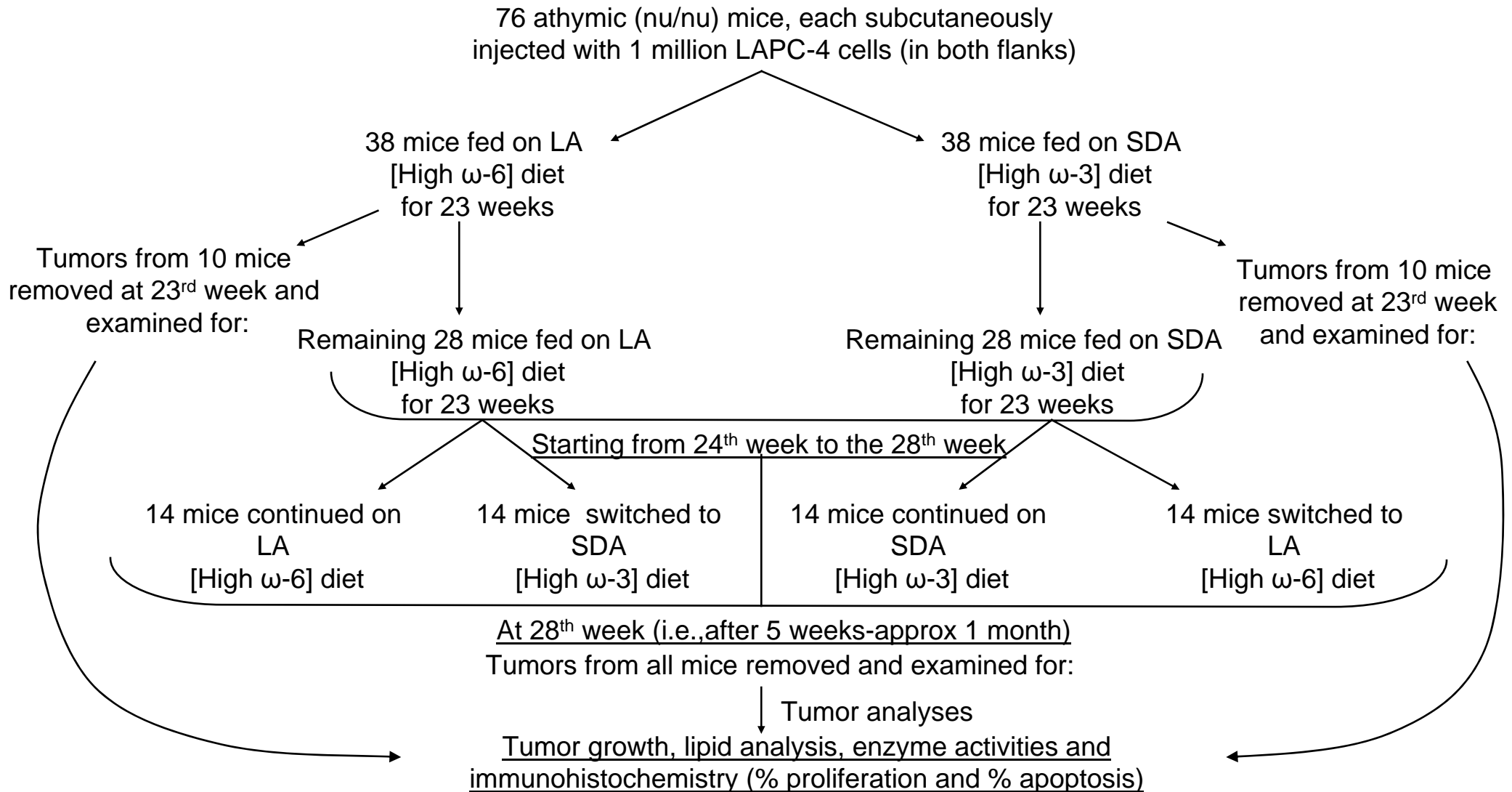
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Figure 1:



**Figure 2:**

Experimental Strategy

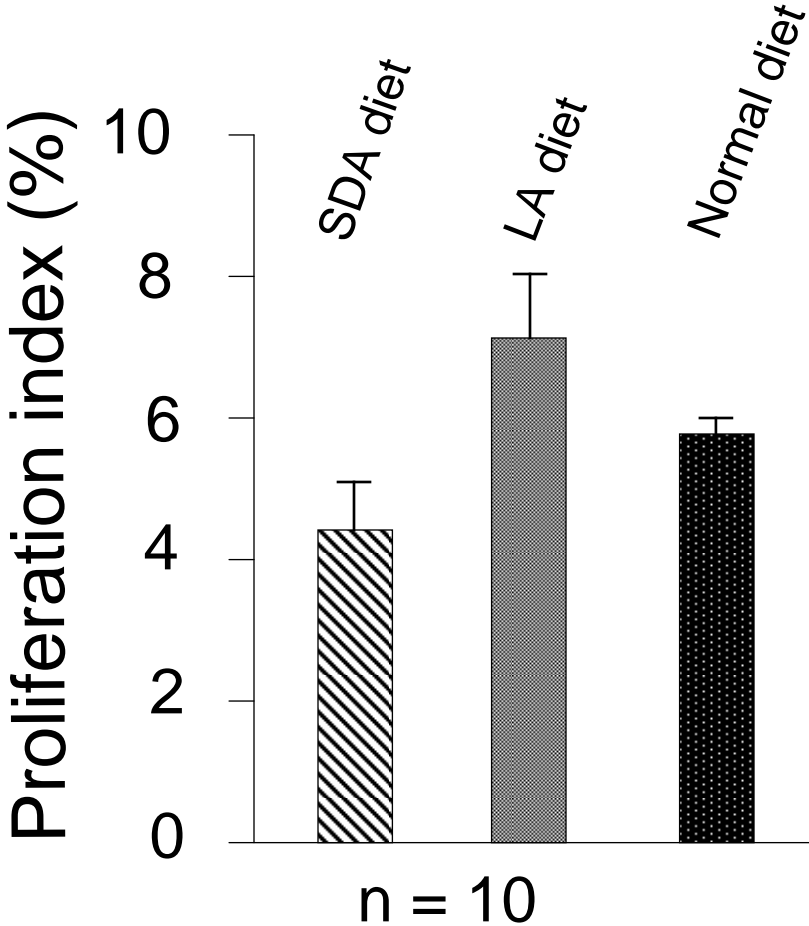


Note: 25 mice similarly injected with LAPC-4 cells were fed with normal (control) diet, tumors from 10 mice removed at 23<sup>rd</sup> week and 10 mice continued on the same diet until 28 weeks; their tumors were similarly analyzed.

Figure 3:

A Week 23

X



Y

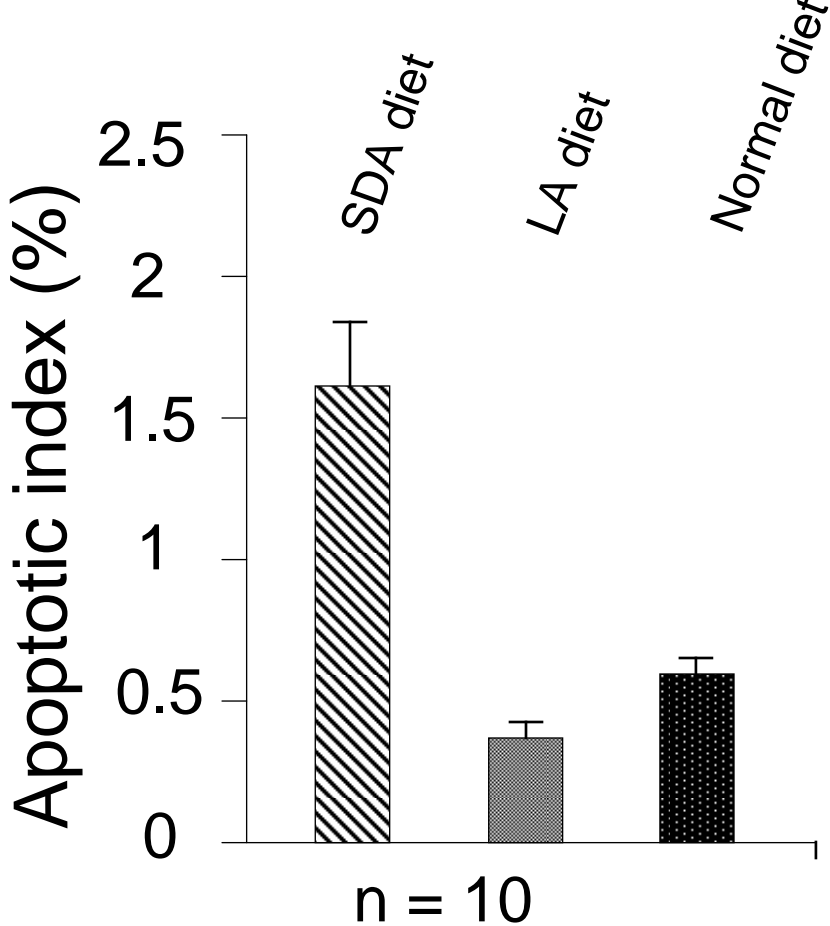
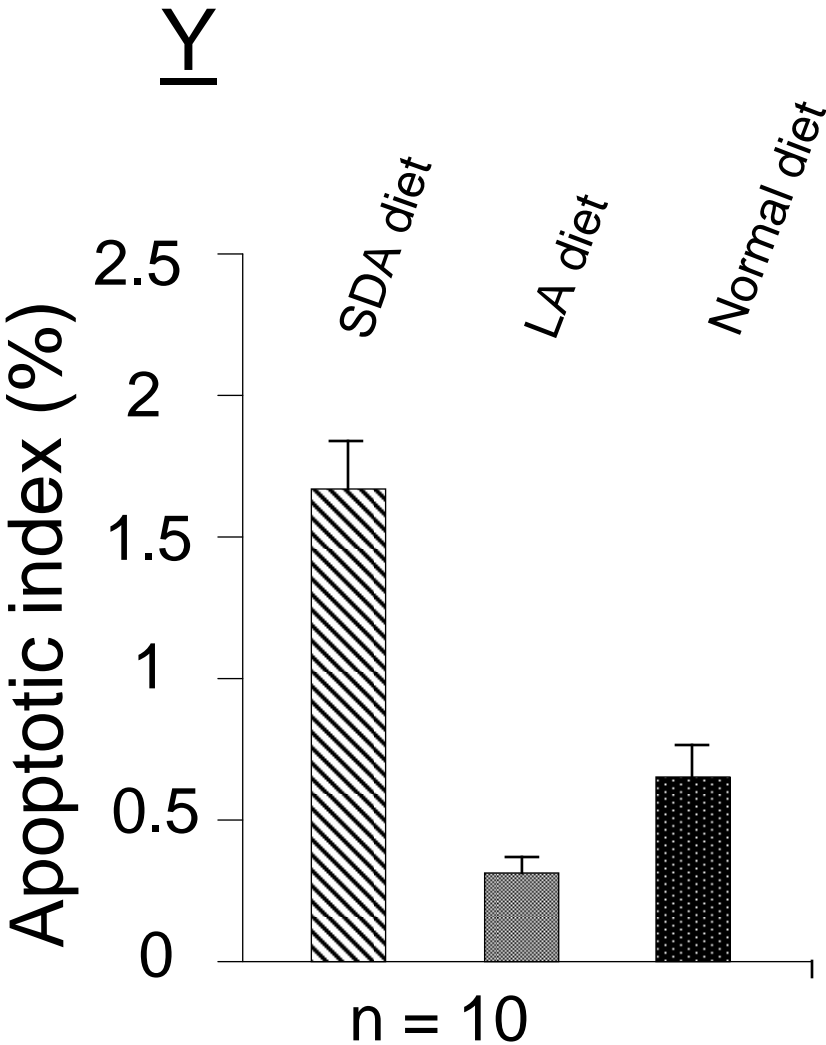
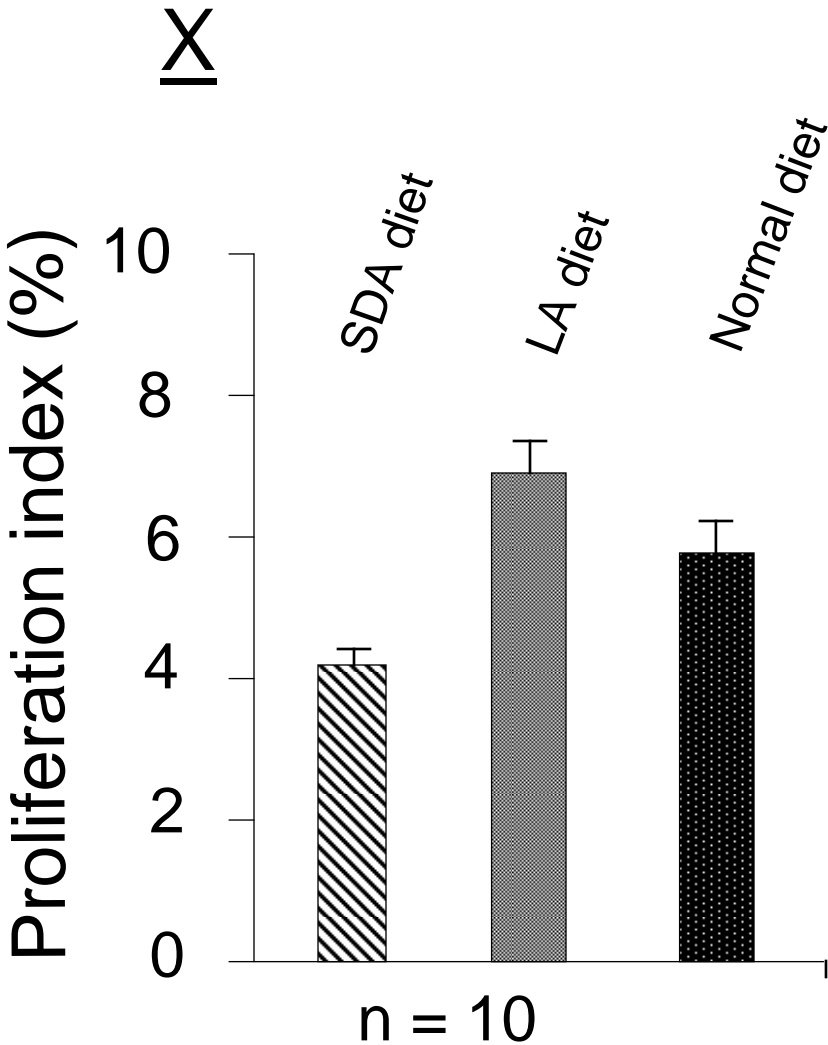


Figure 3:

B Week 28

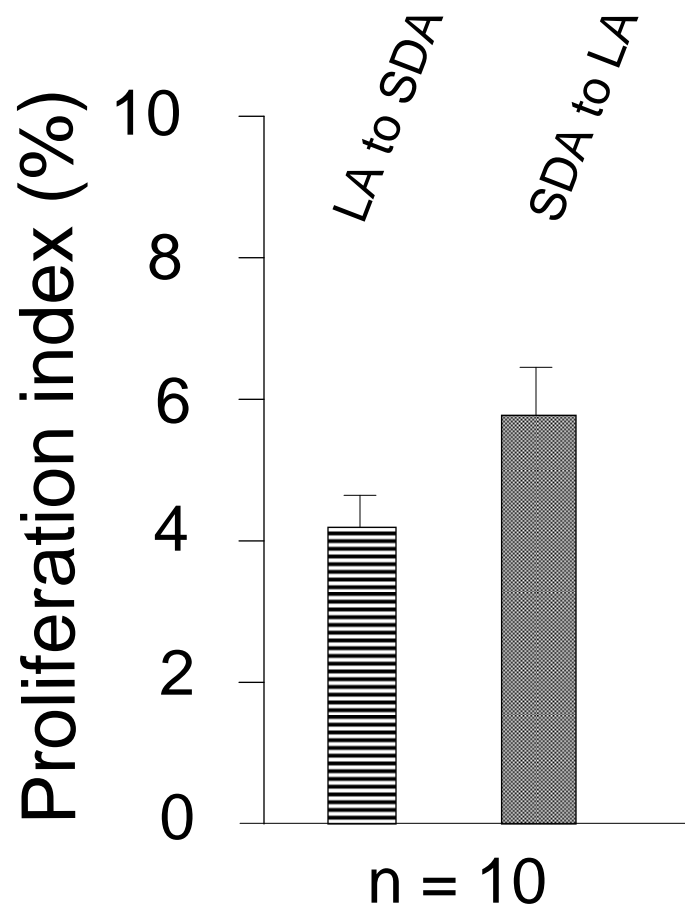


**Figure 3:**

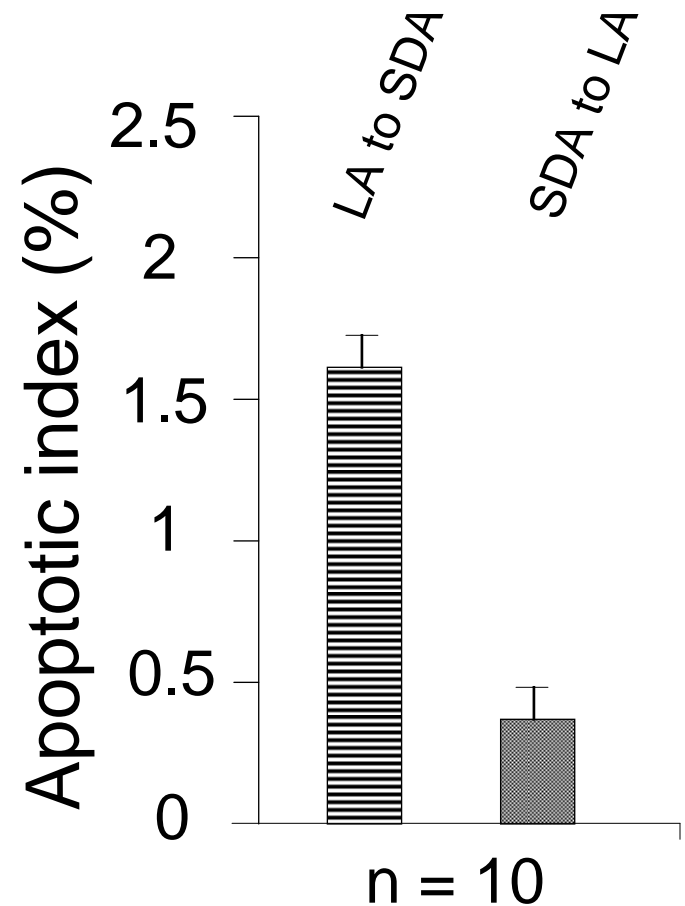
C

Diet switched mice  
(from week 23- to week 28)

X



Y



**Table 1:**

<b>Ingredients<sup>†</sup></b>	<b>Normal</b>	<b>SDA [<math>\omega</math>-3]</b>	<b>LA [<math>\omega</math>-6]</b>
Sucrose	15.0	35.0	35.0
Casein - Vitamin Free	21.0	21.0	21.0
RP Mineral Mix #10 (adds 1.29% fiber)	5.0	5.0	5.0
Powdered Cellulose	3.0	3.0	3.0
RP Vitamin Mix (adds 1.9% sucrose)	2.0	2.0	2.0
Choline Chloride	0.2	0.2	0.2
DL-Methionine	0.2	0.2	0.2
Stearidonic Acid	0.0	15.0	0.0
Safflower Oil	0.0	0.0	15.0
Dextrin	38.6	18.6	18.6
Corn Oil	5.0	0.0	0.0
Lard	10.0	0.0	0.0

A

**Table 2:**

Diet group n= 10 % of total phospholipids	Normal		LA diet		SDA diet		$\omega$ -6 $\rightarrow\omega$ -3	$\omega$ -3 $\rightarrow\omega$ -6
	23 <sup>rd</sup> week	28 <sup>th</sup> week	23 <sup>rd</sup> week	28 <sup>th</sup> week	23 <sup>rd</sup> week	28 <sup>th</sup> week	5 <sup>th</sup> week	5 <sup>th</sup> week
Linoleic acid (LA)	6.2 $\pm$ 0.6	5.9 $\pm$ 0.8	12.2 $\pm$ 0.6	11.7 $\pm$ 0.6	2.2 $\pm$ 0.1	2.4 $\pm$ 0.1	3.2 $\pm$ 0.6	11.4 $\pm$ 0.3
Arachidonic acid (AA)	6.3 $\pm$ 0.3	6.7 $\pm$ 0.4	8.2 $\pm$ 0.6	8.4 $\pm$ 0.5	6.1 $\pm$ 0.1	6.4 $\pm$ 0.3	6.4 $\pm$ 0.6	7.1 $\pm$ 0.7
Eicosapentaenoic acid (EPA)	ND	ND	ND	ND	3.4 $\pm$ 0.2	3.2 $\pm$ 0.3	3.9 $\pm$ 1.2	0.9 $\pm$ 0.4
Docosahexaenoic acid (DHA)	ND	ND	ND	ND	6.2 $\pm$ 0.8	5.9 $\pm$ 1.2	5.8 $\pm$ 1.2	0.4 $\pm$ 0.06

B

Diet group n= 10 % of total phospholipids	Normal		LA diet		SDA diet		$\omega$ -6 $\rightarrow\omega$ -3	$\omega$ -3 $\rightarrow\omega$ -6
	23 <sup>rd</sup> week	28 <sup>th</sup> week	23 <sup>rd</sup> week	28 <sup>th</sup> week	23 <sup>rd</sup> week	28 <sup>th</sup> week	5 <sup>th</sup> week	5 <sup>th</sup> week
Linoleic acid (LA)	5.9 $\pm$ 0.4	5.8 $\pm$ 0.7	12.4 $\pm$ 0.4	12.1 $\pm$ 0.7	2.5 $\pm$ 0.7	2.3 $\pm$ 0.9	3.1 $\pm$ 0.5	11.8 $\pm$ 0.4
Arachidonic acid (AA)	6.1 $\pm$ 0.3	6.2 $\pm$ 0.3	7.8 $\pm$ 0.5*	7.9 $\pm$ 0.7	6.4 $\pm$ 0.2	6.1 $\pm$ 0.5	6.6 $\pm$ 0.8	6.9 $\pm$ 0.8
Eicosapentaenoic acid (EPA)	ND	ND	ND	ND	3.3 $\pm$ 0.3	3.5 $\pm$ 0.1	3.4 $\pm$ 0.5	1.1 $\pm$ 0.2
Docosahexaenoic acid (DHA)	ND	ND	ND	ND	6.4 $\pm$ 0.4	5.7 $\pm$ 0.6	5.9 $\pm$ 1.1	0.6 $\pm$ 0.04



**Table 3:**

Metabolic products Diet group at week 23 (n=10)	13-HODE	15-HETE	15-HEPE	Total prostaglandins
Normal	0.9 ± 0.2	0.1**	0	6.1 ± 0.33
LA diet	2.1 ± 0.2	ND	0	6.2 ± 0.46
SDA diet	ND	ND	1.8 ± 0.06	7.1 ± 0.26
LA to SDA (after 5 weeks time point)	0.2 ± 0.1	ND	1.4 ± 0.03	7.6 ± 0.54
SDA to LA (after 5 weeks time point)	1.8*	ND	0.3 ± 0.03	6.4 ± 0.22

**Table 4:**

Diet group at week 23	% Unsaturated of total phospholipids	% Saturated of total phospholipids	PUFA n-6/n-3	Elongase	Delta -5 desaturase	Delta -6 desaturase	Delta -9 desaturase
Normal	49.4 ± 1.7	42.7 ± 1.5	3.4 ± 1.1	84.1 ± 1.7	7.1 ± 0.6	3.1 ± 0.6	12.7 ± 0.6
LA diet	51.1 ± 1.2	44.1 ± 1.3	9.4 ± 1.1	110.1 ± 1.6	14.4 ± 0.3	7.4 ± 0.5	7.1 ± 0.7
SDA diet	50.2 ± 0.9	43.2 ± 1.1	0.8 ± 0.1	42.2 ± 0.9	0.78 ± 0.06	12.9 ± 1.2	28.7 ± 1.2
Diet group at week 28	% Unsaturated of total phospholipids	% Saturated of total phospholipids	PUFA n-6/n-3	Elongase	Delta -5 desaturase	Delta -6 desaturase	Delta -9 desaturase
Normal	48.7 ± 1.1	43.2 ± 1.7	3.5 ± 1.3	86.1 ± 2.4	6.9 ± 0.8	3.3 ± 0.2	13.0 ± 0.5
LA [High fat, n-6]	52.3 ± 1.1	43.7 ± 1.4	9.2 ± 1.3	115.3 ± 2.3	16 ± 0.5	6.2 ± 0.7	6.2 ± 0.7
LA to SDA (after 5 weeks exposure)	50.1 ± 1.3	42.8 ± 1.3	2.4 ± 0.6	32.3 ± 0.7	0.92 ± 0.04	15.4 ± 2.2	25.4 ± 2.6
SDA diet	49.5 ± 1.4	44.1 ± 1.5	0.9 ± 0.2	37.4 ± 1.7	0.59 ± 0.05	11.7 ± 1.7	27.7 ± 1.7
SDA to LA (after 5 weeks of exposure)	52.6 ± 0.7	43.3 ± 1.1	8.1 ± 0.4	108.1 ± 2.9	12.2 ± 0.01	6.8 ± 0.2	7.2 ± 1.2